Estrogen-Induced Apoptosis of Breast Epithelial Cells Is Blocked by NO/cGMP and Mediated by Extranuclear Estrogen Receptors

Irida Kastrati, Praneeth D. Edirisinghe, Gihani T. Wijewickrama, and Gregory R. J. Thatcher

Department of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, University of Illinois at Chicago, Chicago, Illinois 60612

Estrogen action, via both nuclear and extranuclear estrogen receptors (ERs), induces a variety of cellular signals that are prosurvival or proliferative, whereas nitric oxide (NO) can inhibit apoptosis via caspase *S*-nitrosylation and via activation of soluble guanylyl cyclase to produce cGMP. The action of 17β-estradiol (E_2) at ER is known to elicit NO signaling via activation of NO synthase (NOS) in many tissues. The MCF-10A nontumorigenic, mammary epithelial cell line is genetically stable and insensitive to estrogenic proliferation. In this cell line, estrogens or NOS inhibitors alone had no significant effect, whereas in combination, apoptosis was induced rapidly in the absence of serum; the presence of inducible NOS was confirmed by proteomic analysis. The application of pharmacological agents determined that apoptosis was dependent upon NO/cGMP signaling via cyclic GMP (cGMP)-dependent protein kinase and could be replicated by inhibition of the phosphatidylinositol 3 kinase/serine-threonine kinase pathway prior to addition of E_2. Apoptosis was confirmed by nuclear staining and increased caspase-3 activity in E_2 + NOS inhibitor-treated cells. Apoptosis was partially inhibited by a pure ER antagonist and replicated by agonists selective for extranuclear ER. Cells were rescued from E_2-induced apoptosis after NOS blockade, by NO-donors and cGMP pathway agonists; preincubation with NO donors was required. The NOS and ER status of breast cancer tissues is significant in etiology, prognosis, and therapy. In this study, apoptosis of preneoplastic mammary epithelial cells was triggered by estrogens via a rapid, extranuclear ER-mediated response, after removal of an antiapoptotic NO/cGMP/cGMP-dependent protein kinase signal. **(*Endocrinology* 151: 5602–5616, 2010)**

Nitric oxide (NO), produced by the NO synthase (NOS) family of proteins, modulates a variety of important physiological responses, including cell proliferation, invasion, migration, expression of angiogenic factors, and apoptosis. Regulation of these responses is mediated by activation of the primary NO effector soluble guanylyl cyclase (sGC) to produce the secondary messenger cyclic GMP (cGMP), and by NO-based chemical modifications of proteins and lipids, or reactions with metals and free radicals. NO is a lipophilic, short-lived physiological messenger, argued to diffuse over a wide area (1). On the other hand, there are arguments in favor of the concept of local or spatially defined NO signaling (2–4).

NO steady-state concentration has emerged as a key determinant of biological function (5), whereby lower concentrations promote cell survival and proliferation via cGMP-dependent pathways, and higher levels favor cell cycle arrest, apoptosis, and senescence (6). The latter cytotoxic effects have been associated with activation of cells involved in inflammatory responses and induction of inducible NOS (iNOS) (7). Several studies suggest that high

ISSN Print 0013-7227 ISSN Online 1945-7170
Printed in U.S.A.

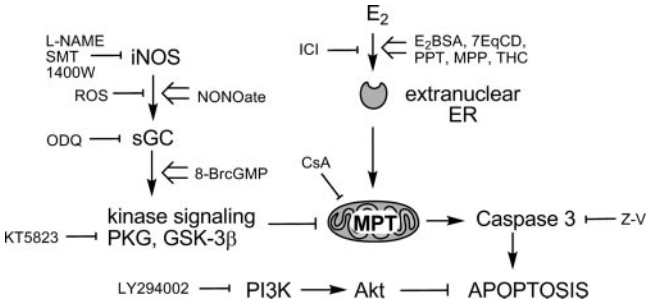
doi: 10.1210/en.2010-0378 Received March 31, 2010. Accepted August 30, 2010.
First Published Online October 13, 2010

Abbreviations: Akt, Serine-threonine kinase; Bcl-2, B-cell lymphoma 2; cGMP, cyclic GMP; CsA, cyclosporine A; DAPI, 4,6′-Diamidino-2-phenylindole; DEA, diethylamine; DMA, desmethylarzoxifene; DMSO, dimethylsulfoxide; E_2, estradiol; EGF, epidermal growth factor; EGFR, EGF receptor; EN, equilenin; eNOS, endothelial NOS; ER, estrogen receptor; FBS, fetal bovine serum; GPR-30, G protein-coupled receptor 30; GSK, glycogen synthase kinase; ICI, ICI 182780; iNOS, inducible NOS; L-NAME, N^G-nitro-*L*-arginine; MPT, mitochondrial permeability transition; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; nNOS, neuronal NOS; NO, nitric oxide; NOS, NO synthase; ODQ, 1H-[1,2,4]oxadiazolo-[4,3-a]quinoxalin-1-one; PI3K, phosphatidylinositol 3 kinase; PKG, cGMP-dependent protein kinase; ROS, reactive oxygen species; sGC, soluble guanylyl cyclase; SERM, selective ER modulator; SFM, serum-free medium; SMT, S-methyl-L-thiocitrulline; SNAP, *S*-nitroso-*N*-acetylpenicillamine; SPE, spermine; TNBC, triple negative breast cancer; Z-VAD-FMK, benzyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethylketone.

NO concentrations generated by exogenous sources or forced overexpression of iNOS may be used to kill tumor cells; paradoxically, iNOS is overexpressed in many tumors, and overexpression of iNOS often correlates with poor prognosis, leading to iNOS inhibition as a proposed target for cancer therapy (8, 9). cGMP-independent posttranslational nitrosation of caspase and pol(ADP-ribose) polymerase are reported as antiapoptotic cell defenses (10, 11). Apoptosis is a fundamental cellular activity to protect against neoplastic development by eliminating genetically damaged cells. Inhibition of spontaneous and metabolically induced apoptosis is one mechanism underlying carcinogenesis, facilitating tumor progression and limiting the effectiveness of cancer treatment.

Observations from clinical trials and epidemiological studies support the hypothesis that estrogen contributes to breast cancer (12–14). In breast cancer epithelial cell lines, there is strong evidence for a prosurvival signal induced by estradiol (E_2) leading to up-regulation of the B-cell lymphoma 2 (Bcl-2) antiapoptotic factor (15, 16). In contrast, in normal cells, inhibition by E_2 of apoptosis induced by oxidative or other stress is documented: for example, in cardiomyocytes, E_2 prevented apoptosis via activation of phosphatidylinositol 3 kinase (PI3K) and serine-threonine kinase (Akt) phosphorylation (17, 18).

MCF-10A immortalized breast epithelial cells play an increasingly important role in studies on chemical carcinogenesis, because the malignant transformation of these "normal" cells can be correlated with genetic changes (19–21). The growth and survival of normal cells in culture is dependent on exogenous factors present in serum, whereas tumor cells have a reduced requirement for such factors and can often survive under serum-free conditions. Growth and survival factors activate a variety of intracellular signal transduction pathways, including antiapoptotic PI3K/Akt and MAPK signaling pathways (22). Inhibition of these kinase pathways is a target for therapy in estrogen sensitive, endocrine resistant, and chemoresistant breast cancer (23). Therefore, it was of interest to study the interplay of estrogen, PI3K/Akt, and NO in serum-deprived MCF-10A cells. Estrogens are not proliferative in MCF-10A cells, and in contrast, in this study were revealed to be apoptotic when PI3K/Akt or NO/cGMP pathways were blocked. Proteomic analysis showed that iNOS was induced at low levels, maintaining a cGMP-dependent cytoprotective brake on estrogen-triggered apoptosis. These data reveal a novel antiapoptotic role for iNOS/NO/cGMP, likely via inhibition of mitochondrial pore transition; in addition to a novel estrogenic, proapoptotic signal via an extranuclear estrogen receptor (ER) in human breast epithelial cells (Scheme 1).



SCHEME 1. Modulation of both the NO/cGMP and PI3K/Akt antiapoptotic signals in MCF-10A human mammary epithelial cells, was elicited by pharmacological intervention using pathway inhibitors (*e.g.* LY294002 and KT5823) or pathway activators (*e.g.* 8-Br-cGMP). Attenuation of the antiapoptotic NO/cGMP signaling pathway revealed the ability of estrogens to elicit apoptosis via extranuclear ER. In the absence of NO/cGMP-mediated control, the activation of this extranuclear ER mediated a rapid estrogenic cell death signal that was blocked by inhibition of MPT or inhibition of caspase-3. For a full description of pharmacological agents, mechanisms of action, and abbreviations used, please see *Materials and Methods*.

Materials and Methods

All chemicals were purchased from Sigma (St. Luis, MO) or Cayman Chemical (Ann Arbor, MI) unless stated otherwise. Pharmacological agents used: 1400W NOS inhibitor [K_i endothelial NOS (eNOS) = 75 μM; K_i iNOS = 0.14 μM; K_i neuronal NOS (nNOS) = 2.0 μM]; 7EqCD extranuclear ER agonist; 8-Br-cGMP membrane-permeable cGMP analog; AG1478 epidermal growth factor (EGF) receptor (EGFR) antagonist; BAY58-2667 (Cinaciguat) sGC activator; cyclosporine A (CsA) immunosupressant blocks mitochondrial permeability transition (MPT); diethylamine (DEA)/NO diazeniumdiolate NO donor ($t_{1/2}$ = 2 min); desmethylarzoxifene (DMA) selective ER modulator (SERM) DMA; E_2-BSA membrane-impermeable BSA protein-conjugated estrogen; equilenin (EN) equine estrogen; G-15 selective G protein-coupled receptor 30 (GPR-30) antagonist; ICI 182780 (ICI) pure antiestrogen; KT5823 cGMP-dependent protein kinase (PKG) inhibitor; menadione redox-cycling reactive oxygen species (ROS) generating *p*-quinone; MPP (methyl-piperidino-pyrazole dihydrochloride selective ERα antagonist); N^G-nitro-*L*-arginine (L-NAME) prodrug of the NOS inhibitor (K_i eNOS = 0.04–0.2 μM; K_i iNOS = 4 μM; K_i nNOS = 0.02–0.2 μM) (24–27); LY294002 PI3K inhibitor; 1H- [1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) sGC inhibitor; PD98059 MAPK/ERK kinase inhibitor; PPT [1,3,5-Tris(4-hydroxyphenyl)-4-propyl-1H-pyrazole selective ERα agonist]; SB203580 p38 MAPK inhibitor; S-methyl-L-thiocitrulline (SMT) NOS inhibitor (K_i eNOS = 11 μM; K_i iNOS = 40 μM; K_i nNOS = 1.2 μM) (28, 29); *S*-nitroso-*N*-acetylpenicillamine (SNAP); spermine (SPE)/NO diazeniumdiolate NO donor ($t_{1/2}$ = 40 min); THC [(R,R)-*cis*-diethyltetrahydro-2,8-chrysenediol ERβ antagonist and ERα partial agonist]; and z-v caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethylketone (Z-VAD-FMK). 7EqCD, BAY58–2667, and G-15 were synthesized by literature procedures and fully characterized (30–32).

Cell culture

All cell culture reagents were purchased from Invitrogen (Carlsbad, CA) unless stated otherwise. MCF-10A cells, originally from the Michigan Cancer Foundation (Rochester, MI),

were sourced as gifts from Mei Lin Chen (University of Illinois at Chicago), Vincent Cryns (Northwestern University, Chicago, IL), or from American Type Culture Collection (ATCC; Manassas, VA). MCF-10A cells were grown in phenol-red-free DMEM/F-12 media supplemented with 100 ng/ml cholera toxin, 10 g/ml insulin, 0.5 g/ml hydrocortisol, 20 ng/ml EGF, 1% 10,000 U penicillin G, 10 mg/ml streptomycin, and 5% heat-inactivated fetal bovine serum (FBS). For drug treatments, MCF-10A cells were treated with compounds as indicated in DMEM/F12 media in the absence of serum and other growth factors/supplements; unless otherwise indicated, cells were pretreated with L-NAME for 1 h. Antibodies for Western blotting were purchased from Cell Signal (Danvers, MA). BT20 cells and HCC38 cells were a kind gift from Vincent Cryns (Northwestern University). BT20 cells were grown in MEM with Earle's salts and L-glutamine supplemented with 10% FBS, 1 mM sodium pyruvate, 1× nonessential amino acids, 1.5 g/liter sodium bicarbonate and 1% 10,000 U penicillin G, and 10 mg/ml streptomycin. HCC38 cells, originally from ATCC, were grown in RPMI 1640 media supplemented with 10% heat-inactivated FBS.

3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay

At 20–30% confluence, MCF-10A cells were treated in triplicates in 12-well plates. Experiments were repeated in a 96-well plate format at similar low seeding cell density with similar results. At 24-h intervals, assay was performed with MTT (M2128; Sigma-Aldrich, St. Louis, MO) according to the manufacturer's protocol. Similarly, MTS [(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] (CellTiter 96 Aqueous; Promega, Madison, WI) assay was used to measure cell viability. Data are reported as mean ± SEM or SD analyzed by one-way ANOVA with Tukey's multiple comparison test using GraphPad Prism version 4.00 for Windows, GraphPad Software (GraphPad, San Diego, CA).

Immunoenrichment and detection of iNOS

MCF-10A cells were treated with dimethylsulfoxide (DMSO) vehicle as indicated in DMEM/F12 media in the absence of serum and other growth factors/supplements. Cells were washed in PBS, resuspended in lysis buffer (no. 9803; Cell Signal) containing 1 mM phenylmethylsulfonylfluoride for 5 min, mixed, and centrifuged at 12,000 × *g* for 10 min; 1 ml of cell lysate was collected and treated with NaN_3 and 2 ml of PBS. Immunoprecipitation of iNOS was performed by the addition of a mixture of 0.25 μl biotinylated iNOS antibody (Signet, Norwell, MA). Samples were then rotated at room temperature for 12 h followed by the addition of 4 μl T1 streptavidin coated magnabeads and rotated for an additional 2 h. Beads were washed three times with 10 mM NH_4CO_3 (pH 8.0), and the iNOS protein was eluted using 25 μl of a mixture of 75% acetonitrile and 1% trifluoroacetic acid in water. The acid neutralized, concentrated proteins were digested with trypsin. The peptide mixture (30 μl, 10 μg enriched proteins) was injected onto a reversed phase column (75 μm × 150 mm Zorbax SB300 C-18; Agilent Technologies, Santa Clara, CA) connected to a Dionex Ultimate 3000 HPLC system and a Thermo Finnigan LTQ-FT mass spectrometer equipped with a nanospray interface. The samples were chromatographed using a binary solvent system consisting of A, 0.1% formic acid and 5% acetonitrile; and B, 0.1% formic acid and 95% acetonitrile at a flow rate of 200 nl/min. A gradient was run from 15% B to 55% B over 60 min. The mass spectrometer was operated in positive ion mode with the trap set to data-dependent MS/MS acquisition mode. Data analysis was carried out using the MassMatrix software platform (33, 34). The library searching and *de novo* interpretation identified the detected proteins from the individual peptides. The results for all proteins detected were collected and listed by protein name, detected peptide sequence(s), and search score.

Western blot analysis

MCF-10A cells were treated with compounds as indicated; pretreatment with the different inhibitors varied from 30 min to 1 h. Cells were washed in PBS, resuspended in lysis buffer (no. 9803; Cell Signal) containing 1 mM phenylmethylsulfonylfluoride for 5 min, mixed, and centrifuged at 12,000 × *g* for 10 min. Protein concentration was measured in supernatants using the Bradford Assay kit (Bio-Rad Laboratories, Hercules, CA). Equal aliquots of total protein samples (20 μg per lane) were electrophoresed on a 4–12% Bis-Tris polyacrylamide gel, transferred to polyvinylidene fluoride membranes (Invitrogen), and blotted using antibodies to *p*Akt, Akt, *p*-glycogen synthase kinase (GSK)-3β, ERα, eNOS, nNOS (Cell Signal), and iNOS (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). β-Actin was used as a control for loading and transfer. The blotted proteins were visualized using the enhanced chemiluminescence detection system (Fisher Scientific, Itasca, IL) and quantitated using AlphaEaseFC software.

Caspase-3 activity

Caspase-3 activity was measured with Sigma colorimetric caspase-3 kit using DEVD-*p*-nitroanilide (DEVD-pNA) as substrate according to the manufacturer's instructions. Caspase inhibitor Ac-DEVD-CHO (2 mM) was used as a negative control. Results are expressed relative to control caspase-3 activity.

4,6′-Diamidino-2-phenylindole (DAPI) staining

MCF-10A cells were grown (10^6 cells/ml) on each of eight wells on a sterile Nunc chambered cover glass and incubated for 48 h at 37 C with 5% CO_2 containing the above media. MCF-10A cells were treated with E_2 1 nM, L-NAME 5 μM, DMSO as vehicle control, and E_2+L-NAME where L-NAME was administered as a pretreatment for 30 min. Then treated cells were washed with PBS and fixed with 4% paraformaldehyde for 30 min. Cells were rinsed two times with PBS and mounted with 0.2 ml of DAPI hydrochloride containing mounting media (Vector Laboratories, Inc., Burlingame, CA). The coverslip was sealed with nail polish to prevent drying and movement under microscope. Imaging was performed with a Zeiss LSM510 laser-scanning confocal microscope (Zeiss, Oberkochen, Germany) with the detector gain adjusted to eliminate the background autofluorescence. The fluorescence signal from DAPI staining was monitored with a 345-nm UV laser and a 440-nm band pass filter. A ×63 (1.2 numerical aperture) water immersion objective was used for all experiments.

Results

NOS inhibition facilitates a death signal induced by E_2 but not by survival/growth factors

Mammary epithelial cells can respond to serum starvation by undergoing apoptosis. However, MCF-10A

cells have been reported to respond by undergoing apoptosis only when confluent and plated at high density (35, 36). For example, in serum-free medium (SFM), the increase in apoptotic nuclei at 48 h was reported to be inhibited by addition of EGF, insulin, or extracellular matrix components, changes accompanied by modulation of proapoptotic (Bax) and antiapoptotic factors (Bcl-2). Here, MCF-10A cells plated at low density were observed to show a typical response to serum starvation and growth factor withdrawal in the presence of vehicle using the MTT assay to measure cell viability (Fig. 1A). Addition of IGF restored cell viability independent of NOS inhibition (Fig. 1A). Addition to the SFM of E_2 was also without effect on cell viability (Fig. 1B). Similarly, without effect was the NOS inhibitor L-NAME (5 μM). However, after inhibition of NOS activity by preincubation with L-NAME for 30 min, the addition of E_2 (1 nM) triggered a loss of cell viability not seen with either agent alone (Fig. 1B). To determine whether NOS activity was essential for mediating a general survival pathway, MCF-10A cells were grown in SFM in the presence of the EGFR antagonist, AG1478 (Fig. 1C). Antagonism of the proliferative EGF signal led to the expected reduction in cell viability without dependence on NOS inhibition, whereas the death signal initiated by E_2+L-NAME was additive with the effects of AG1478.

Inhibition of PI3K/Akt signaling facilitates the E_2 death signal

Signal transduction via the PI3K/Akt kinase cascade is known to provide a cellular survival message that may be induced by E_2 or IGF-I (37, 38). Inhibition of PI3K/Akt signaling in MCF-10A cells using LY294002 (5 μM) facilitated the cell death signal elicited by E_2 (Fig. 1D), although LY294002 alone elicited a more modest loss of cell viability. Signaling via p38 MAPK is a pathway associated with caspase induction and has been reported to mediate the proapoptotic effects of NO (39) and to be opposed by an NO-induced antiapoptotic MAPK/ERK signal (40). The MAPK/ERK pathway is normally associated with a proliferative or prosurvival signal, and in MCF-7 cells, rapid activation of ERK is caused both by addition of exogenous NO donors (5) and by the action of estrogen at membrane-associated ER (41). Inhibition of the MAPK/ERK pathway using PD98059 caused cell death independent of E_2 (Fig. 1E). Inhibition of p38 MAPK signaling with SB203580 facilitated a weaker E_2-induced death signal, again independent of NOS inhibition (Fig. 1F).

E_2 rapidly induces apoptosis in the presence of NOS blockade

To confirm the nature of the cell death observed, MCF-10A cells were stained with DAPI dye under similar conditions to those used to derive the data presented in Fig. 1B. Cells exposed to E_2 after preincubation with L-NAME showed the typical cell morphology associated with apoptotic cell death: blebbing, loss of symmetry, shrinkage, and nuclear fragmentation (Fig. 2). Of note was the rapid onset of these cellular changes, within the first 30 min after exposure to E_2.

Caspase-3 activity is increased by E_2 and amplified by NOS inhibition

Caspase-3 is a critical executioner of apoptosis in the caspase cascade; activation requiring proteolytic degradation of the inactive 32-kDa zymogen into the activated p17 and p12 subunits. The combination (E_2+L-NAME) significantly increased activation of caspase-3, which was inhibited by the selective caspase-3 inhibitor, Ac-DEVD-CHO (Fig. 3A). Immunoassay of the inactive zymogen of caspase-3 was in accord with caspase activity measurements; the combination (E_2+L-NAME) decreased the remaining protein levels of the inactive zymogen (Fig. 3B). Notably, inactive zymogen immunoreactivity was decreased after only 15 min. GSK-3β is a downstream element of the PI3K/Akt cell survival pathway, and its activity can be inhibited by Akt-mediated phosphorylation at Ser9. Serum-starved MCF-10A cells treated with DMSO vehicle showed increased GSK-3β phosphorylation relative to control cells, but the combination (E_2+L-NAME) increased *p*-GSK-3β levels significantly (Fig. 3B).

Evidence for ER and iNOS in MCF-10A cells

Weak immunoreactivity toward ERα was observed in MCF-10A cells, independent of cell treatment (Fig. 3B). Immunoreactivity to eNOS and nNOS was not observed (data not shown), but immunoreactivity to iNOS was observed (Fig. 3B). A mass spectrometry proteomic analysis was carried out on MCF-10A cells incubated in SFM to confirm the presence of iNOS; the results were positive (65% sequence coverage; 28 protein identification score). The full proteomic analysis is shown in Supplemental data, published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org.

Alternative NOS inhibitors support apoptosis induced by E_2

The guanidine NOS inhibitor, 1400W, is a selective and irreversible inhibitor of iNOS (42, 43), whereas the thiocitrulline NOS inhibitor, SMT, is nNOS selective (28, 29). However, the inhibition potency for NOS inhibitors has been reported to be highly tissue and species dependent. All NOS inhibitors were observed to facilitate E_2-induced apoptosis compatible with these molecules functioning via NOS inhibition rather than any idiosyncratic action of the inhibitor itself (Fig. 4).

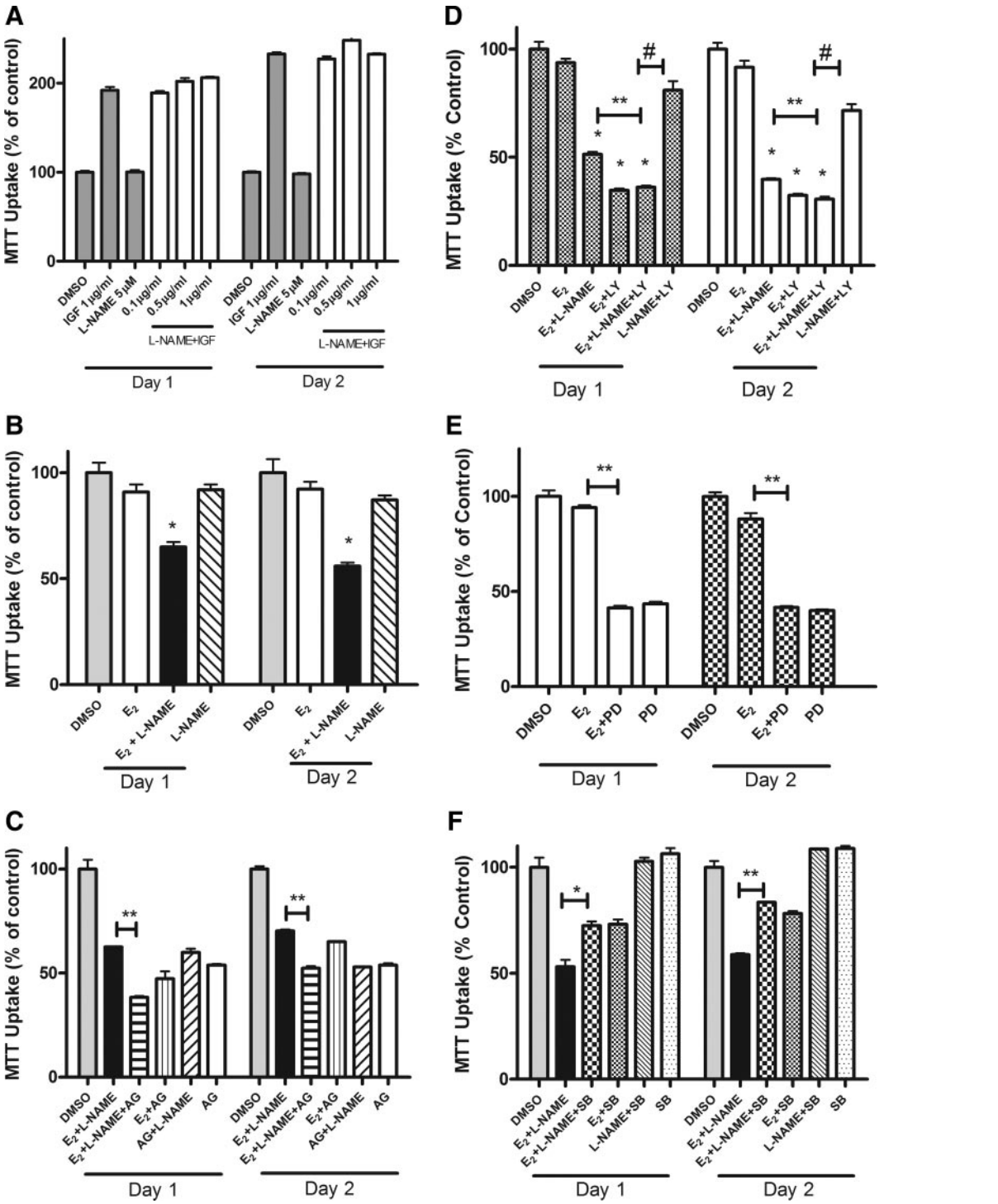


FIG. 1. Effect of growth factors, E_2, L-NAME, and inhibitors of the MAPK/ERK, PI3K/Akt, and p38MAPK pathways on viability of serum-deprived MCF-10A cells. A, The prosurvival factor IGF in the presence and absence of NOS inhibitor (L-NAME) (5 μM) supports growth; B, E_2 (1 nM) in the presence of L-NAME decreases cell viability but has no effect in the absence of NOS inhibitor. *, $P < 0.001$ E_2+L-NAME *vs.* E_2 or L-NAME alone. C, Growth was inhibited by the EGFR antagonist tyrphostin [AG1478 (AG), 5 μM] that further decreased cell viability by the E_2+L-NAME combination. **, $P < 0.001$. L-NAME was added 30 min before hormone, factor, or antagonist. D, Inhibition of the Akt pathway by PI3K inhibitor LY294002 (LY) (5 μM) + E_2 replicated the actions of L-NAME+E_2. *, $P < 0.001$ for vehicle (DMSO) *vs.* E_2+L-NAME, E_2+LY, and E_2+LY+L-NAME; **, $P < 0.001$ for E_2+L-NAME *vs.* E_2+LY+L-NAME; #, $P < 0.001$ for L-NAME+LY *vs.* E_2+LY+L-NAME. E, Inhibition of MAPK/ERK signaling using the MAPK/ERK kinase inhibitor PD 98059 (PD) (5 μM) resulted in reduced MCF-10A cell viability independent of E_2 (1 nM). **, $P < 0.001$ for E_2+PD compared with E_2 or vehicle. F, The p38 MAPK inhibitor SB203580 (SB) (5 μM) facilitated the death signal elicited by E_2 but to a lesser extent than L-NAME and LY and showed no additive effect with L-NAME (5 μM). *, $P < 0.05$; **, $P < 0.001$. All pathway inhibitors were added 30 min before addition of E_2. Data obtained by MTT assay show mean and SEM analyzed by ANOVA with Tukey *post hoc* test.

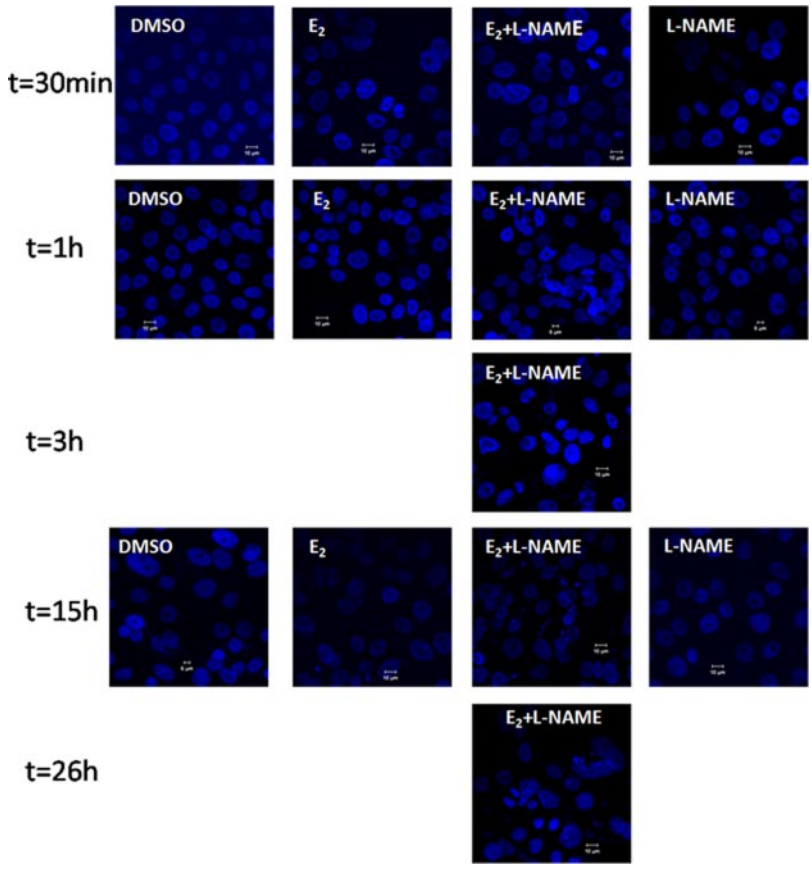


FIG. 2. Nuclear DNA DAPI staining of MCF-10A cells at different time points showing morphology associated with apoptosis in the presence of E_2 (1 nM) + L-NAME (5 μM) in SFM. Treated cells showed typical markers of nuclear condensation associated with apoptosis as early as 30 min after addition of E_2. Data show representative images from analysis of triplicate cell cultures.

Effects of estrogens and antiestrogens in presence of NOS blockade

In the presence of L-NAME (5 μM), the concentration response for E_2-induced apoptosis indicated an $EC_{50} < 1$ nM (Fig. 4C). The clinically relevant equine estrogen, EN, replicated the apoptotic activity of E_2 in the presence of both NOS blockade and inhibition of PI3K/Akt signaling (Fig. 5A). The SERM DMA, a ligand for both ERα and ERβ (44, 45), produced no significant effect in the presence of NOS or PI3K inhibition (Fig. 5B). However, the "pure" antiestrogen, ICI did partially inhibit E_2-induced apoptosis (Fig. 5C). In contrast, G-15, a selective antagonist of GPR-30 (sometimes referred to as G protein-coupled ER) did not inhibit the E_2+L-NAME-mediated cell death signal (31) (data not shown). The classical actions of ER as a nuclear receptor and transcription factor have been differentiated from nonclassical activity by use of commercially available, membrane impermeable probes, such as E_2-BSA (46–48). Extended incubation of E_2-BSA in cell culture leads to degradation to BSA and E_2; therefore, the E_2 conjugate, 7EqCD, a stable, extranuclear ER agonist was also employed (30, 47). Both 7EqCD (Fig. 5B) and E_2-BSA (Fig. 5D) at nanomolar concentration induced cell death *in simile* with E_2, providing strong evidence that the ER-mediated pathway is extranuclear. Selective agonists of classical ERα and ERβ signaling triggered the estrogenic cell death signal in MCF-10A cells (Fig. 5D); the lack of selectivity and the replication of E_2 activity support a nonclassical, extranuclear ER pathway.

Inhibition of E_2-induced apoptosis by NOS is cGMP-dependent mediated by PKG

NO-induced antiapoptotic mechanisms have been proposed, which are either cGMP dependent or cGMP independent, the last often via protein *S*-nitrosylation (signaling) or caspase *S*-nitrosation (enzyme inhibition). E_2 was observed to elicit an ERβ-dependent apoptotic signal in colon cancer cells, which was counteracted by NO, purportedly via caspase *S*-nitrosation (49). In primary cerebellar granule cells, sustained attenuation of constitutive NOS activity by L-NAME (1 mM) induced apoptosis via inhibition of PI3K/Akt and GSK-3β; the actions of L-NAME were mimicked by the sGC inhibitor ODQ (100 μM) that inhibits production of cGMP (50). ODQ-induced apoptosis has been reported in uterine epithelial cells and human ovarian cancer cells (51). Apoptotic activity in uterine epithelial cells has also been reported for the PKG inhibitor KT5823 (52). Both ODQ (10 μM) and KT5823 (5 μM) were observed fully to mimic the activity of L-NAME in facilitating the apoptotic signal induced by E_2 (Fig. 6, A and B). Blockade of NO/cGMP/PKG signaling is therefore required to reveal the E_2 apoptotic signal in MCF-10A cells.

Restoring blocked NO/cGMP signaling

The diazeniumdiolates NO-donors SPE/NO and DEA/NO and the nitrosothiol SNAP were studied to determine whether exogenous NO was able to counteract the effects of L-NAME in MCF-10A cells. L-NAME was added as a pretreatment, 30 min before addition of E_2 and NO donor, as in the experiments described above. Surprisingly, none of the NO-donor treatments were able fully to protect cells from the apoptotic signal of E_2 (Fig. 6C); indeed, the data suggested that micromolar concentrations of NO donors caused a further decrease in cell viability. Finally, the sGC activator BAY58-2667 was examined, but again, no restoration of cell viability was observed (Fig. 6C). To explain these observations, it was

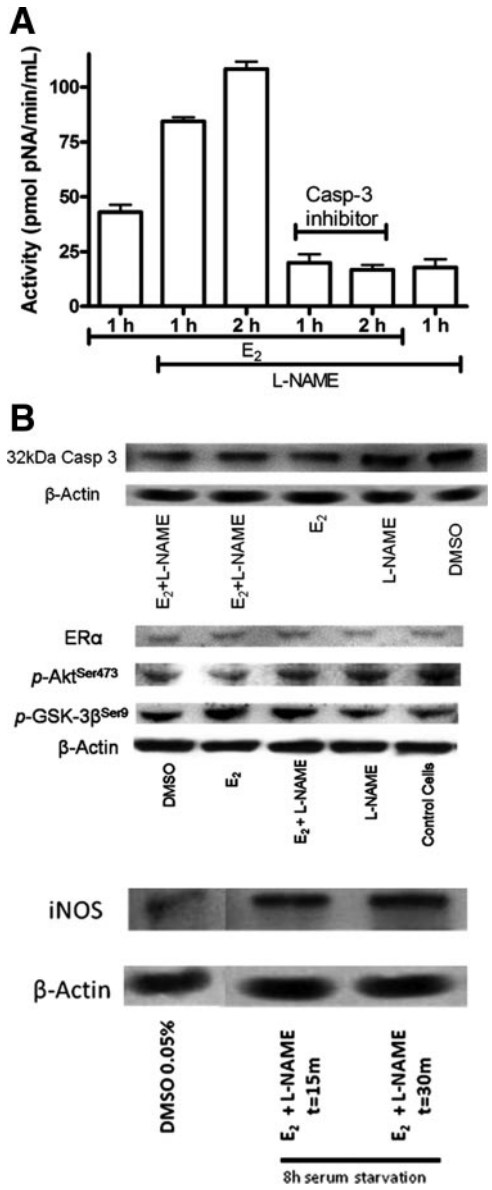


FIG. 3. Caspase-3 activation and levels of caspase and other proteins in MCF-10A cells in SFM. A, Increased caspase-3 activity after treatment with E_2+L-NAME measured 1 or 2 h after addition of E_2 (1 nM). Cell lysate supernatants were incubated overnight with caspase-3 peptide substrate Ac-DEVD-pNA with or without Ac-DEVD-CHO inhibitor measuring product formation at $\lambda = 405$ nm. Data show mean and SD. B, Immunoassay of protein levels from MCF-10A cell lysates. *Upper*, activation of inactive caspase-3 zymogen (32 kDa) to activated p17 and p12 subunits; cells showed significantly higher levels of inactive caspase-3 in control cells and those treated with L-NAME alone. *Middle*, GSK-3β is a downstream element of the PI3K/Akt cell survival pathway, the activity of which is inhibited by Akt-mediated phosphorylation at Ser9; GSK-3β phosphorylation was decreased in control and L-NAME treated cells. *Lower*, Western blotting showed ERα immunoreactivity in all cell treatments; immunoprecipitation with iNOS antibody and Western blotting clearly showed presence of this isoform.

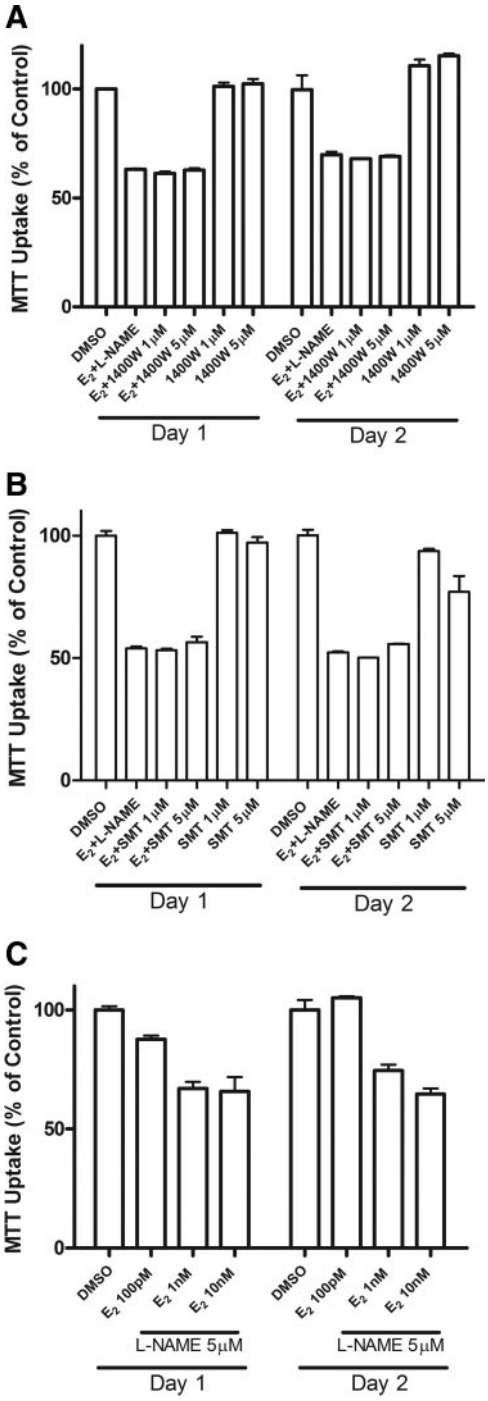


FIG. 4. Comparison of NOS inhibitors in facilitation of E_2-induced cell death. Cells pretreated with the selective iNOS inhibitor 1400W (A) and the selective nNOS inhibitor SMT (B) were assayed in the presence or absence of E_2 (1 nM) and compared with L-NAME (5 μM). E_2+NOS inhibitor treatments at all inhibitor concentrations tested are significantly different ($P < 0.001$) from either agent alone. C, Cell death elicited by E_2 in the presence of L-NAME was concentration dependent. All treatments with NOS inhibitor + E_2 were significant ($P < 0.001$) with the exception of treatments with $E_2 = 100$ pM. Data show mean and SEM.

postulated that prior addition of NO would be required to restore cellular NO, thereby attenuating the ER-mediated apoptotic signal induced by E_2. Serum-deprived MCF-10A cells were pretreated with L-NAME for various incubation times before addition of E_2; preincubation was required to observe E_2-induced cell death (Fig. 7A). Therefore, NO donors were preincubated with L-NAME before

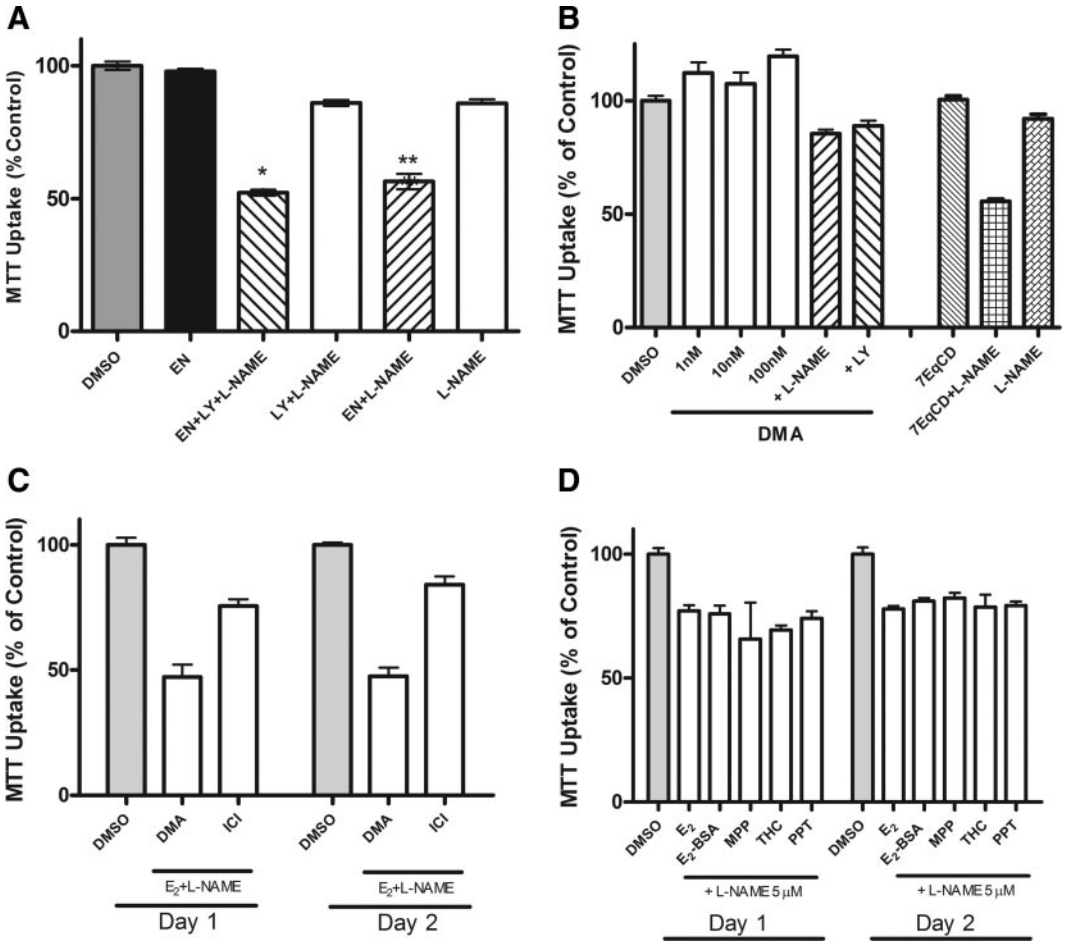


FIG. 5. Effects of estrogens and antiestrogens on cell viability in MCF-10A cells subject to NOS or PI3K/Akt inhibition. A, Cell viability 24 h after treatment with the equine estrogen EN (1 nM) in cells pretreated for 30 min with L-NAME or LY294002 (LY) (5 μM each). *, $P < 0.001$ *vs.* LY+L-NAME; **, $P < 0.001$ *vs.* L-NAME. B, Cell viability 24 h after treatment with the SERM, DMA (10 nM), in the presence or absence of L-NAME, or LY pretreatment (5 μM each) compared with treatment with extranuclear ER ligand, 7EqCD (1 nM) in the presence and absence of L-NAME (5 μM). No significant differences in treatment groups ($P > 0.05$) except 7EqCD+L-NAME ($P < 0.01$). C, Antiestrogenic effects of DMA (1 μM) and ICI (1 μM) on the cell death signal elicited by E_2 (1 nM) in the presence of L-NAME (5 μM). D, Ability of various estrogens (all 1 nM) to elicit death response in MCF-10A cells pretreated with L-NAME (5 μM). Membrane-impermeable BSA protein-conjugated estrogen (E_2-BSA); selective ERα antagonist MPP (methyl-piperidino-pyrazole dihydrochloride); ERβ antagonist and ERα partial agonist THC; selective ERα agonist PPT [1,3,5-Tris(4-hydroxyphenyl)-4-propyl-1H-pyrazole]. No significant difference between estrogen+L-NAME treatment groups was observed ($P > 0.05$). The combination (estrogen+L-NAME) is significantly different ($P < 0.001$) from either agent alone. Data show mean and SEM.

addition of E_2 (Fig. 7B), and a similar experiment was carried out using the membrane-permeable cGMP analog, 8-Br-cGMP at concentrations comparable with published studies (Fig. 7C) (53). These data show that restoration of NO/sGC/cGMP signaling, by exogenous NO or a cGMP analog, is able to block the actions of E_2. However, the estrogenic signal is rapid, and once the cell is committed to apoptosis, restoration of NO/cGMP signaling is ineffective.

Depletion of NO by ROS

One of the consequences of ROS generation is to reduce NO concentrations (6). Therefore, the redox-cycling, ROS donor, menadione was examined in MCF-10A cells to determine whether NO depletion by ROS could mimic the effects of NO depletion by NOS inhibition (Fig. 7D). Low concentrations of menadione were used, because high concentrations of ROS are well known independently to induce cell toxicity. In the absence of L-NAME, preincubation of MCF-10A cells with menadione was able to facilitate the E_2-induced apoptotic signal. In control experiments, the effects of menadione on E_2+L-NAME treatment were examined: menadione had no effect when preincubated with L-NAME. Therefore, the ability of menadione to mimic the effects of L-NAME may be ascribed to the depletion of NO by ROS.

The E_2+L-NAME apoptotic signal was abrogated by caspase inhibition and mitochondrial pore stabilization

Apoptosis via the extrinsic pathway is mediated at least in part via loss of mitochondrial membrane integrity and caspase activation (54). MPT, an increase in the perme-

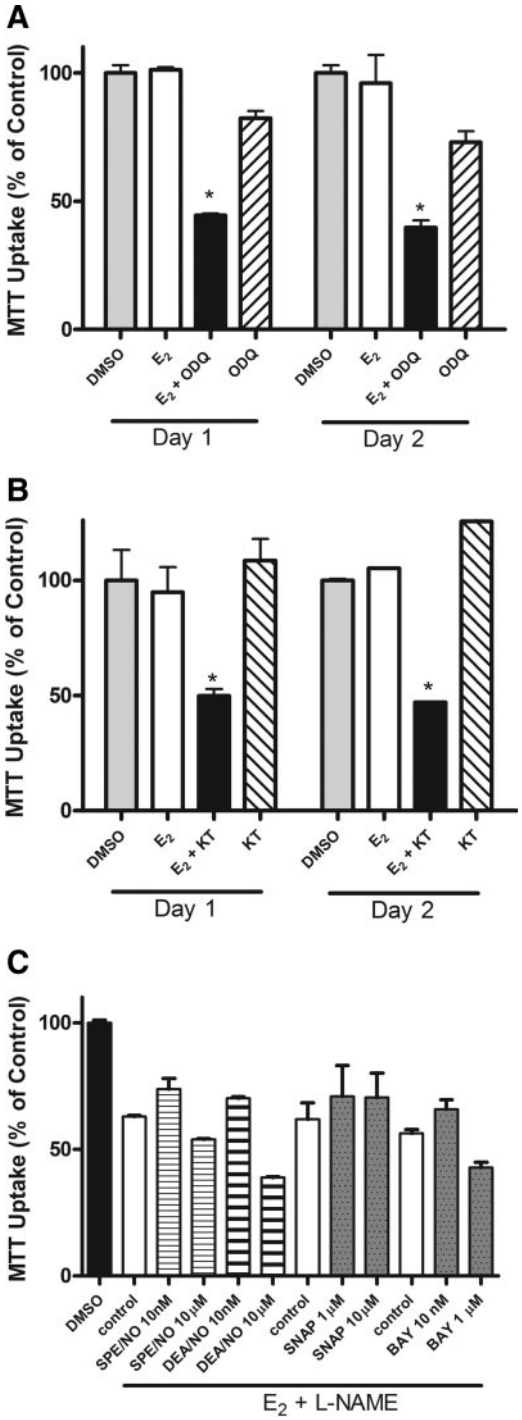


FIG. 6. MCF-10A cell viability in the presence of NO/cGMP/PKG pathway modulators and E_2. Inhibition of sGC by ODQ (10 μM) (A) and inhibition of PKG by KT5823 (KT) (5 μM) (B) added as pretreatment for 30 min before addition of E_2 (1 nM). *, $P < 0.001$ *vs.* ODQ alone and *vs.* KT alone or E_2 alone. C, Cells were pretreated for 30 min with L-NAME (5 μM) followed by addition of E_2 (1 nM) with varied concentrations of pathway modulators for 24 h: true NO-donor diazeniumdiolates SPE/NO and DEA/NO, the *S*-nitrosothiol SNAP, and the NO-independent sGC activator BAY58–2667 (BAY). Data show mean and SD.

ability of the mitochondrial membrane to cytochrome C and other factors, results from opening of MPT pores, leading to mitochondrial swelling and cell death (55). CsA prevents MPT by blocking pore opening, thus inhibiting mitochondrial cytochrome C release and inhibiting apoptosis mediated via MPT. The effect on serum-starved MCF-10A cell viability after E_2+L-NAME treatment was studied in the presence of CsA and the membrane-permeable irreversible pan-caspase inhibitor Z-VAD-FMK. Both treatments were shown to inhibit the death signal induced by E_2 in the presence of NOS signal blockade (Fig. 8). CsA is protective in liver and other tissues, however, it is reported to have a relatively narrow concentration range of efficacy and to be toxic at higher concentration (56), consistent with our observations (Fig. 8A). The antiapoptotic effect of Z-VAD-FMK observed in MCF-10A cells at lower concentrations was lost at higher concentrations (Fig. 8B), which has precedent in the literature (57). Therefore, the observed proapoptotic estrogenic signal appears to be mediated by MPT and is confirmed to involve caspase activation.

NOS inhibition reveals an estrogenic cell death signal in other breast epithelial and cancer cell lines

MCF-10A cells are an immortalized, genetically stable cell line often used as a model for preneoplastic breast epithelial cells (58). These cells are nonproliferative in response to E_2 and usually classified as ER negative, although ER protein and mRNA have been reported in these cells (59). In addition to MCF-10A cells, the BT20 and HCC38 cell lines have been used as models of triple negative breast cancer (TNBC), immunohistochemically classified as lacking progesterone receptor, human epithelial growth factor receptor-2, and nuclear ERα (60, 61). Both BT20 and HCC38 cell lines in SFM responded to the combination of L-NAME and E_2 in a similar way manifesting the estrogenic apoptotic signal in the presence of NOS blockade (Fig. 8, C and D). As expected, ERα positive MCF-7 cells in SFM responded to the proliferative signal of E_2. Moreover, this signal was not perturbed by L-NAME (data not shown).

Discussion

ERα negative and TNBC

Inhibition of metabolically or hormonally induced apoptosis is a mechanism that may contribute to carcinogenesis. The link between estrogen and the development and proliferation of breast cancer is well documented. In several ERα(+) breast cancer epithelial cell lines, there is evidence for an antiapoptotic, prosurvival signal induced by E_2 via classical ER signaling, leading to up-regulation of the Bcl-2 antiapoptotic factor (15, 16, 62, 63). However, in the clinical arena, before the development of ta-

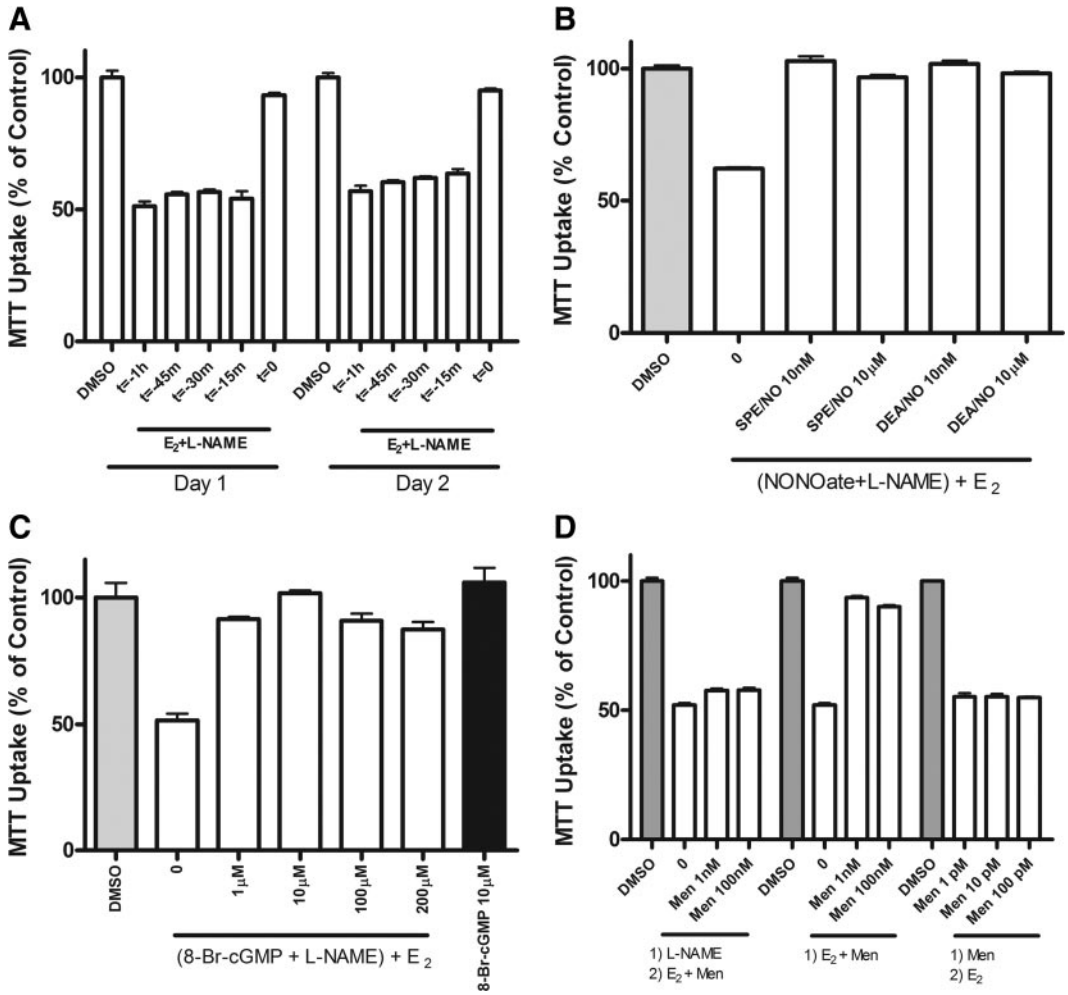


FIG. 7. Importance of timing in blockade of NO/cGMP pathway and restoration of signaling. A, The effect on MCF-10A cell viability of shortening the preincubation time with L-NAME (5 μM) was studied with addition of E_2 (1 nM) at t = 0. A 15-min preincubation with L-NAME is significantly different from E_2+L-NAME cotreatment ($P < 0.001$). Diazeniumdiolate NO donors (B) and the membrane-permeable cGMP analog, 8-Br-cGMP (C), restored NO signaling in the presence of L-NAME when added to cells at the start of the 30-min preincubation period inhibiting the cell death signal elicited by addition of E_2 at t = 0. D, The ROS-generating quinone, menadione (Men), was added to serum-starved MCF-10A cells at different time points: 1) coincident with E_2 after preincubation with L-NAME; 2) coincident with E_2; and 3) without L-NAME, but 30 min before addition of E_2. All E_2+Men treatments are significantly different ($P < 0.001$) from E_2 or Men alone, and they replicate the E_2+L-NAME cell viability. Separate controls were performed for each set of experiments and cell viability measured at 24 h. Data show mean and SD.

moxifen, high-dose estrogen was used to induce tumor regression of hormone-dependent breast cancer in postmenopausal women (64). Although the pathways by which estrogen induces apoptosis are not completely known, the response of normal and malignant mammary cells to estrogens and the relationship to ER status is clearly important in cancer therapy and prevention. In this study, mammary epithelial cells in SFM were observed to undergo estrogen-induced apoptosis mediated by extranuclear ER after inhibition of NO/cGMP/PKG or PI3K/Akt signaling pathways (Scheme 1).

TNBC is an aggressive type of breast cancer with prevalence that is much higher in minority populations and for which there is no optimal therapeutic treatment. However, TNBC does not describe a homogenous group of patients or tumors, moreover, the cell lines often used as models for TNBC are frequently reported to respond to E_2. TNBC tissues and cell lines may be positive for ERβ and its isoforms, extranuclear ER, and GPR-30. For example, in the HCC38 ER(−) cell line, E_2 and E_2-BSA upregulated protein kinase C α, which was correlated with tumor severity (65); in the ER(−) HC11 nonmaligant mouse mammary epithelial cell line, an ERβ-mediated apoptotic signal was identified (66); and, in the classic MDA-MB-231 ER(−) cell line, E_2 activated PI3K/Akt (67). Activation of kinase cascades is a hallmark of rapid, nonclassical estrogen signaling via extranuclear ER and is observed in TNBC cells. ER may be trafficked between cytoplasm, membrane, and nucleus (68), and in addition, GPR-30 is argued to be a cytoplasmic receptor for E_2 (69). In the present work, three TNBC cell lines in SFM were observed to be susceptible to an estrogenic apoptotic sig-

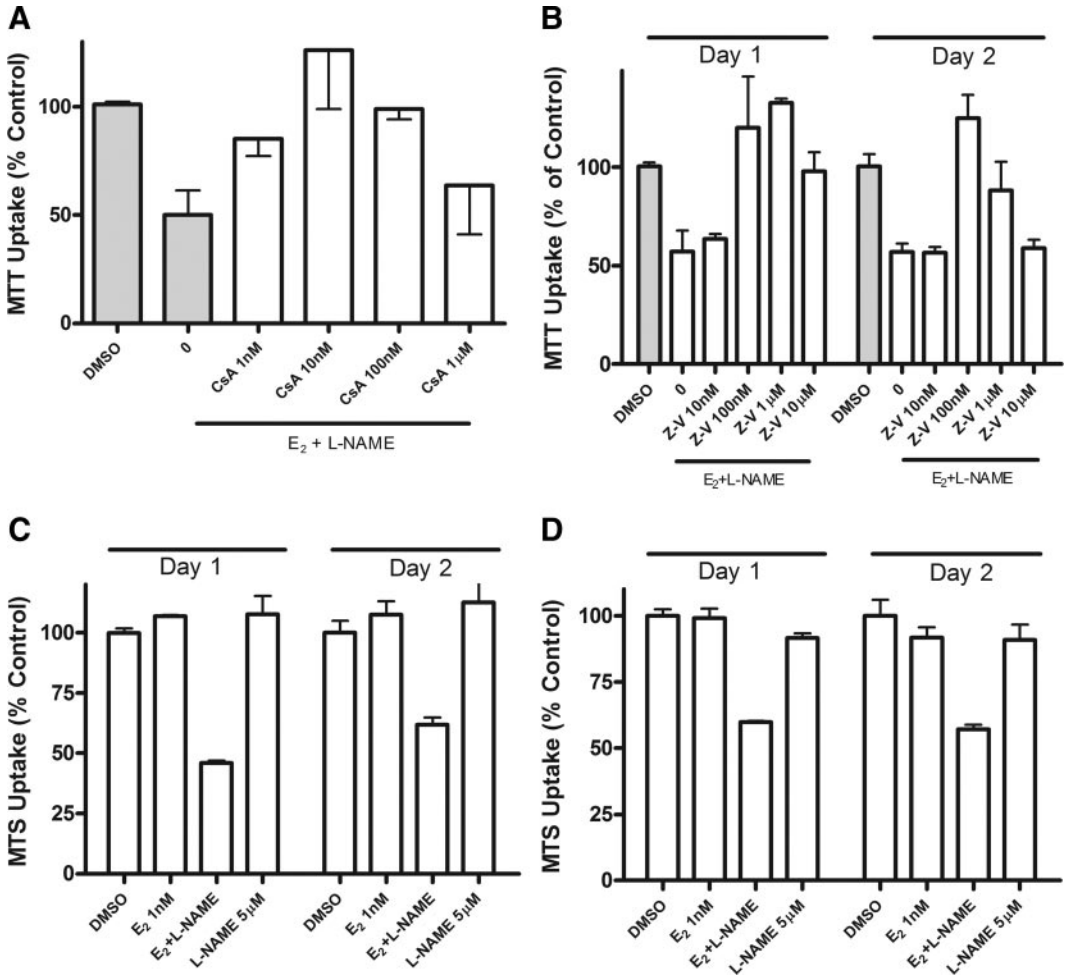


FIG. 8. The apoptotic actions of E_2 in the presence of NOS blockade are observed in other mammary epithelial cells and in MCF-10A cells are inhibited by antiapoptotic factors. A, The MTP inhibitor, CsA inhibited the reduction in MCF-10A cell viability induced by E_2 (1 nM) + L-NAME (5 μM) measured after 24 h. Cells were preincubated with CsA and L-NAME for 30 min. B, The tripeptide membrane-permeable caspase inhibitor, Z-VAD-FMK, was added to cells and preincubated for 30 min with L-NAME before addition of E_2. Addition of CsA (1 nM) and Z-VAD-FMK (100 nM) restores the reduced cell viability ($P < 0.001$). C, In BT20 cells, E_2 (1 nM) in the presence of L-NAME decreases cell viability but had no effect in the absence of NOS inhibitor. *, $P < 0.001$ E_2+L-NAME *vs.* E_2 or L-NAME alone. D, In HCC38 cells, E_2 (1 nM) in the presence of L-NAME decreases cell viability but had no effect in the absence of NOS inhibitor. *, $P < 0.001$ E_2+L-NAME *vs.* E_2 or L-NAME alone. Cell viability in C and D was measured by MTS. Data show mean and SD (A, C, and D) or SEM (B).

nal, and in MCF-10A cells, this signal was shown to be mediated by extranuclear ER rather than GPR-30.

Linkages between estrogenic and NO signaling

Activation and up-regulation of eNOS and nNOS by E_2 has been reported (70–72); eNOS activation by E_2 was blocked by inhibition of PI3K, by ICI, and by L-NAME (73). Scaffolding of ERα to MAPK/ERK, PI3K/Akt, and eNOS has been proposed to provide estrogen-induced activation of all three pathways (74). Marino and co-workers (75) have reported that E_2 binding to ERβ was associated with activation of p38 MAPK and a proapoptotic signal, whereas overproduction, or addition of exogenous NO, was associated with inhibition of E_2-induced apoptosis by *S*-nitrosylation of either caspase-3 or ERβ (49). The proapoptotic signal induced by E_2 in MCF-10A cells was observed on inhibition of NOS or inhibition of PI3K/Akt signaling, but inhibition of MAPK/ERK, or p38 MAPK, pathways gave a different response. The present data do not define if PI3K/Akt is upstream of NOS, as has been reported for activation of eNOS. However, both intact NO and PI3K/Akt signaling blocked E_2-induced apoptosis. A correlative relationship between Akt phosphorylation and elevated iNOS has been reported in breast cancer (76).

NO as antiapoptotic factor

Several cGMP-dependent and cGMP-independent mechanisms have been proposed for induction of pro- and antiapoptotic pathways by NO (77), the latter including inhibition of executor caspase activity by nitrosation of the active site cysteine residue (78). Cytokine-induced hepatocyte apoptosis was blocked by a cGMP analog that also activated PI3K/Akt in these cells. However, inhibition

of PI3K by LY294002 had no effect on apoptosis (79). Correlations of apoptosis induced by growth factor deprivation or cytokine application in rat hepatocytes treated with NO donors (NONOate and SNAP), ODQ, 8-Br-cGMP, KT5823, and caspase-3 inhibitor (Ac-DEVD-CHO), led to the conclusion that the antiapoptotic activity of NO was partially cGMP dependent (80). Apoptosis of PC-12 pheochromocytoma cells induced by serum starvation was observed to be inhibited by 8-Br-cGMP, Ac-DEVD-CHO, and SNAP, the latter effect being blocked by ODQ and by KT5823 (53). Comparable results were reported in primary cell culture of rat motor neurons after trophic factor brain-derived neurotrophic factor starvation, in which endogenous NO was reduced by L-NAME (1 mM), leading to increased apoptosis (81). Studies in primary cerebellar granule cells have added mechanistic information (50); after differentiation, these cells induce robust NOS activity, inhibition of which by L-NAME (1 mM) was reported to lead to apoptosis, which was probed using the usual tools (NONOate, Ac-DEVD-CHO, and 8-Br-cGMP), and shown to be cGMP dependent and mediated by PI3K/Akt/GSK-3, and not by MAPK or c-Jun N-terminal kinase cascades. In the presence of L-NAME over 96 h, a time-dependent reduction in phospho-Akt and phospho-GSK-3β was reported. In neurons, the prosurvival actions of NO appear to be mediated by activation of PI3K/Akt elevating phospho-GSK-3β (82). In ovarian cancer cells, linkage of NO/cGMP-mediated inhibition of apoptosis to tumorigenesis was drawn: apoptosis was induced by ODQ (30–100 μM) and partially attenuated by 8-Br-cGMP (1 mM) via activation of caspase-3 (51). In this setting, the prosurvival activity was ascribed to stabilization and activation of the p53 oncogene by NO/cGMP signaling. Finally, cell death of glucose-deprived hepatocytes was observed to be prevented by SNAP and 8-Br-cGMP, an effect attenuated by the PKG inhibitor, KT5823, and mediated via inhibition of mitochondrial membrane depolarization (83). In MCF-10A cells, perturbation of the NO/cGMP/PKG signaling pathway, using L-NAME, KT5823, and ODQ, categorically demonstrated that the antiapoptotic activity of NOS was cGMP dependent and resulted in inhibition of caspase-3 activation and was likely mediated by inhibition of MPT.

NO plays a crucial role in inducing and maintaining mitochondrial biogenesis (84). Markers of mitochondrial function are depressed in all tissues of eNOS knockout mice; evidence from cell cultures indicates that NO/cGMP signaling activates peroxisome proliferator-activated receptor γ, leading to mitochondrial biogenesis, activity that can be partially replicated by exogenous SNAP (1–300 μM) and 8-Br-cGMP (1 mM) and can be partially blocked by ODQ and inhibited by L-NAME. Of significance is the further observation that NO/cGMP signaling inhibits MPT, an essential event in release of proapoptotic factors, including cytochrome C (56); and similar observations have been ascribed to exogenous NO (85). In MCF-10A cells, DEA/NO and SPE/NO blocked cell death induced by E_2+L-NAME when cells were preincubated with NO donor before addition of E_2. A similar antiapoptotic signal was elicited by inhibition of caspase-3 and by inhibition of MPT by CsA. The rapid induction of apoptosis by E_2+L-NAME is consistent with the role of mitochondria in transducing and amplifying a proapoptotic signal (86).

A prosurvival role for iNOS

The potential for ROS-induced mitochondrial DNA damage to contribute to carcinogenesis has been recognized (87). Superoxide, the primary ROS produced by mitochondria reacts with NO to form peroxynitrite, releasing cytotoxic oxidizing radicals (NO_2 and CO_3^-). Such nitroxidative stress is commonly associated with high fluxes of NO from inflammatory up-regulation of iNOS. In the present work, the presence of low levels of iNOS in MCF-10A cells under stress from serum deprivation was confirmed by proteomic analysis. Thus, in this system at least, a low NO flux from iNOS is able to modulate cell survival of breast epithelial cells under stress. Furthermore, menadione as a cellular ROS source, at very low concentrations, inhibited this cell survival pathway, most likely by trapping of NO by superoxide derived from menadione redox cycling.

In normal human mammary epithelium, both iNOS and eNOS are expressed (88). Both the ER and iNOS status of tissues in breast cancer is of current interest in assessing prognostic markers and in determining therapeutic strategies, in TNBC in particular. In the present study, cells that would normally be classified as ER(−) expressed low levels of iNOS under serum deprivation, which maintained an antiapoptotic NO/cGMP/PKG-mediated inhibition of MPT, blocking an estrogenic apoptotic signal mediated via extranuclear ER and caspase-3 activation (Scheme 1). Whether the antiapoptotic PI3K/Akt signal is intrinsic or parallel to this pathway was not determined. In normal tissues and cells, there is evidence for prosurvival roles for NO and for apoptotic signals from E_2. However, the conjunction of these pathways in mammary epithelial cells, and the central roles of iNOS and extranuclear ER, is to the best of our knowledge novel.

Acknowledgments

Address all correspondence and requests for reprints to: Gregory R. J. Thatcher, Department of Medicinal Chemistry and Phar-

macognosy, College of Pharmacy, University of Illinois at Chicago, Chicago, Illinois 60612. E-mail: thatcher@uic.edu.

This work was supported by National Institutes of Health Grant CA102590.

Disclosure Summary: The authors have nothing to disclose.

References

1. **Lancaster Jr JR** 1997 A tutorial on the diffusibility and reactivity of free nitric oxide. Nitric Oxide 1:18–30
2. **Iwakiri Y, Satoh A, Chatterjee S, Toomre DK, Chalouni CM, Fulton D, Groszmann RJ, Shah VH, Sessa WC** 2006 Nitric oxide synthase generates nitric oxide locally to regulate compartmentalized protein S-nitrosylation and protein trafficking. Proc Natl Acad Sci USA 103: 19777–19782
3. **Barouch LA, Harrison RW, Skaf MW, Rosas GO, Cappola TP, Kobeissi ZA, Hobai IA, Lemmon CA, Burnett AL, O'Rourke B, Rodriguez ER, Huang PL, Lima JA, Berkowitz DE, Hare JM** 2002 Nitric oxide regulates the heart by spatial confinement of nitric oxide synthase isoforms. Nature 416:337–339
4. **Kim SF, Huri DA, Snyder SH** 2005 Inducible nitric oxide synthase binds, S-nitrosylates, and activates cyclooxygenase-2. Science 310: 1966–1970
5. **Thomas DD, Espey MG, Ridnour LA, Hofseth LJ, Mancardi D, Harris CC, Wink DA** 2004 Hypoxic inducible factor 1α, extracellular signal-regulated kinase, and p53 are regulated by distinct threshold concentrations of nitric oxide. Proc Natl Acad Sci USA 101:8894–8899
6. **Thomas DD, Ridnour LA, Isenberg JS, Flores-Santana W, Switzer CH, Donzelli S, Hussain P, Vecoli C, Paolocci N, Ambs S, Colton CA, Harris CC, Roberts DD, Wink DA** 2008 The chemical biology of nitric oxide: implications in cellular signaling. Free Radic Biol Med 45:18–31
7. **Stuehr DJ, Santolini J, Wang ZQ, Wei CC, Adak S** 2004 Update on mechanism and catalytic regulation in the NO synthases. J Biol Chem 279:36167–36170
8. **Ambs S, Merriam WG, Ogunfusika MO, Bennett WP, Ishibe N, Hussain SP, Tzeng EE, Geller DA, Billiar TR, Harris CC** 1998 p53 and vascular endothelial growth factor regulate tumor growth of NOS2-expressing human carcinoma cells. Nat Med 4:1371–1376
9. **Pervin S, Chaudhuri G, Singh R** 2010 NO to breast: when, why and why not? Curr Pharm Des 16:451–462
10. **Kim YM, Bombeck CA, Billiar TR** 1999 Nitric oxide as a bifunctional regulator of apoptosis. Circ Res 84:253–256
11. **Yu Z, Kuncewicz T, Dubinsky WP, Kone BC** 2006 Nitric oxide-dependent negative feedback of PARP-1 trans-activation of the inducible nitric-oxide synthase gene. J Biol Chem 281:9101–9109
12. **Key T, Appleby P, Barnes I, Reeves G** 2002 Endogenous sex hormones and breast cancer in postmenopausal women: reanalysis of nine prospective studies. J Natl Cancer Inst 94:606–616
13. **Banks E, Canfell K** 2009 Invited commentary: hormone therapy risks and benefits–the Women's Health Initiative findings and the postmenopausal estrogen timing hypothesis. Am J Epidemiol 170: 24–28
14. **Prentice RL, Chlebowski RT, Stefanick ML, Manson JE, Langer RD, Pettinger M, Hendrix SL, Hubbell FA, Kooperberg C, Kuller LH, Lane DS, McTiernan A, O'Sullivan MJ, Rossouw JE, Anderson GL** 2008 Conjugated equine estrogens and breast cancer risk in the Women's Health Initiative clinical trial and observational study. Am J Epidemiol 167:1407–1415
15. **Wang TT, Phang JM** 1995 Effects of estrogen on apoptotic pathways in human breast cancer cell line MCF-7. Cancer Res 55:2487–2489
16. **Perillo B, Sasso A, Abbondanza C, Palumbo G** 2000 17β-Estradiol inhibits apoptosis in MCF-7 cells, inducing bcl-2 expression via two estrogen-responsive elements present in the coding sequence. Mol Cell Biol 20:2890–2901
17. **Kim JK, Pedram A, Razandi M, Levin ER** 2006 Estrogen prevents cardiomyocyte apoptosis through inhibition of reactive oxygen species and differential regulation of p38 kinase isoforms. J Biol Chem 281:6760–6767
18. **Patten RD, Pourati I, Aronovitz MJ, Baur J, Celestin F, Chen X, Michael A, Haq S, Nuedling S, Grohe C, Force T, Mendelsohn ME, Karas RH** 2004 17β-Estradiol reduces cardiomyocyte apoptosis in vivo and in vitro via activation of phospho-inositide-3 kinase/Akt signaling. Circ Res 95:692–699
19. **Santen R, Cavalieri E, Rogan E, Russo J, Guttenplan J, Ingle J, Yue W** 2009 Estrogen mediation of breast tumor formation involves estrogen receptor-dependent, as well as independent, genotoxic effects. Ann NY Acad Sci 1155:132–140
20. **Burdick AD, Ivnitski-Steele ID, Lauer FT, Burchiel SW** 2006 PYK2 mediates anti-apoptotic AKT signaling in response to benzo-[a]pyrene diol epoxide in mammary epithelial cells. Carcinogenesis 27:2331–2340
21. **Marella NV, Malyavantham KS, Wang J, Matsui S, Liang P, Berezney R** 2009 Cytogenetic and cDNA microarray expression analysis of MCF10 human breast cancer progression cell lines. Cancer Res 69:5946–5953
22. **Dudek H, Datta SR, Franke TF, Birnbaum MJ, Yao R, Cooper GM, Segal RA, Kaplan DR, Greenberg ME** 1997 Regulation of neuronal survival by the serine-threonine protein kinase Akt. Science 275: 661–665
23. **Ghayad SE, Vendrell JA, Larbi SB, Dumontet C, Bieche I, Cohen PA** 2010 Endocrine resistance associated with activated ErbB system in breast cancer cells is reversed by inhibiting MAPK or PI3K/Akt signaling pathways. Int J Cancer 126:545–562
24. **Furfine ES, Harmon MF, Paith JE, Garvey EP** 1993 Selective inhibition of constitutive nitric oxide synthase by L-NG-nitroarginine. Biochemistry 32:8512–8517
25. **Pollock JS, Förstermann U, Mitchell JA, Warner TD, Schmidt HH, Nakane M, Murad F** 1991 Purification and characterization of particulate endothelium-derived relaxing factor synthase from cultured and native bovine aortic endothelial cells. Proc Natl Acad Sci USA 88:10480–10484
26. **Klatt P, Schmidt K, Brunner F, Mayer B** 1994 Inhibitors of brain nitric oxide synthase. Binding kinetics, metabolism, and enzyme inactivation. J Biol Chem 269:1674–1680
27. **Garvey EP, Tuttle JV, Covington K, Merrill BM, Wood ER, Baylis SA, Charles IG** 1994 Purification and characterization of the constitutive nitric oxide synthase from human placenta. Arch Biochem Biophys 311:235–241
28. **Narayanan K, Griffith OW** 1994 Synthesis of L-thiocitrulline, L-homothiocitrulline, and S-methyl-L-thiocitrulline: a new class of potent nitric oxide synthase inhibitors. J Med Chem 37:885–887
29. **Erdal EP, Litzinger EA, Seo J, Zhu Y, Ji H, Silverman RB** 2005 Selective neuronal nitric oxide synthase inhibitors. Curr Top Med Chem 5:603–624
30. **Kim HY, Sohn J, Wijewickrama GT, Edirisinghe P, Gherezghiher T, Hemachandra M, Lu PY, Chandrasena RE, Molloy ME, Tonetti DA, Thatcher GR** 2010 Click synthesis of estradiol-cyclodextrin conjugates as cell compartment selective estrogens. Bioorg Med Chem 18:809–821
31. **Dennis MK, Burai R, Ramesh C, Petrie WK, Alcon SN, Nayak TK, Bologa CG, Leitao A, Brailoiu E, Deliu E, Dun NJ, Sklar LA, Hathaway HJ, Arterburn JB, Oprea TI, Prossnitz ER** 2009 In vivo effects of a GPR30 antagonist. Nat Chem Biol 5:421–427
32. **Stasch JP, Schmidt P, Alonso-Alija C, Apeler H, Dembowsky K, Haerter M, Heil M, Minuth T, Perzborn E, Pleiss U, Schramm M, Schroeder W, Schröder H, Stahl E, Steinke W, Wunder F** 2002 NO- and haem-independent activation of soluble guanylyl cyclase: molecular basis and cardiovascular implications of a new pharmacological principle. Br J Pharmacol 136:773–783
33. **Xu H, Freitas MA** 2007 A mass accuracy sensitive probability based

scoring algorithm for database searching of tandem mass spectrometry data. BMC Bioinformatics 8:133
34. **Xu H, Freitas MA** 2009 MassMatrix: a database search program for rapid characterization of proteins and peptides from tandem mass spectrometry data. Proteomics 9:1548–1555
35. **Merlo GR, Basolo F, Fiore L, Duboc L, Hynes NE** 1995 p53-Dependent and p53-independent activation of apoptosis in mammary epithelial cells reveals a survival function of EGF and insulin. J Cell Biol 128:1185–1196
36. **Merto GR, Cella N, Hynes NE** 1997 Apoptosis is accompanied by changes in Bcl-2 and Bax expression, induced by loss of attachment, and inhibited by specific extracellular matrix proteins in mammary epithelial cells. Cell Growth Differ 8:251–260
37. **Kennedy SG, Wagner AJ, Conzen SD, Jordán J, Bellacosa A, Tsichlis PN, Hay N** 1997 The PI 3-kinase/Akt signaling pathway delivers an anti-apoptotic signal. Genes Dev 11:701–713
38. **Ahmad S, Singh N, Glazer RI** 1999 Role of AKT1 in 17β-estradiol- and insulin-like growth factor I (IGF-I)-dependent proliferation and prevention of apoptosis in MCF-7 breast carcinoma cells. Biochem Pharmacol 58:425–430
39. **Ghatan S, Larner S, Kinoshita Y, Hetman M, Patel L, Xia Z, Youle RJ, Morrison RS** 2000 p38 MAP kinase mediates bax translocation in nitric oxide-induced apoptosis in neurons. J Cell Biol 150:335–347
40. **Kim SJ, Ju JW, Oh CD, Yoon YM, Song WK, Kim JH, Yoo YJ, Bang OS, Kang SS, Chun JS** 2002 ERK-1/2 and p38 kinase oppositely regulate nitric oxide-induced apoptosis of chondrocytes in association with p53, caspase-3, and differentiation status. J Biol Chem 277:1332–1339
41. **Chang M, Peng KW, Kastrati I, Overk CR, Qin ZH, Yao P, Bolton JL, Thatcher GRJ** 2007 Activation of estrogen receptor-mediated gene transcription by the equine estrogen metabolite, 4-methoxyequilenin, in human breast cancer cells. Endocrinology 148:4793–4802
42. **Garvey EP, Oplinger JA, Furfine ES, Kiff RJ, Laszlo F, Whittle BJ, Knowles RG** 1997 1400W is a slow, tight binding, and highly selective inhibitor of inducible nitric-oxide synthase in vitro and in vivo. J Biol Chem 272:4959–4963
43. **Furfine ES, Harmon MF, Paith JE, Knowles RG, Salter M, Kiff RJ, Duffy C, Hazelwood R, Oplinger JA, Garvey EP** 1994 Potent and selective inhibition of human nitric oxide synthases. Selective inhibition of neuronal nitric oxide synthase by S-methyl-L-thiocitrulline and S-ethyl-L-thiocitrulline. J Biol Chem 269:26677–26683
44. **Qin Z, Kastrati I, Chandrasena RE, Liu H, Yao P, Petukhov PA, Bolton JL, Thatcher GRJ** 2007 Benzothiophene selective estrogen receptor modulators with modulated oxidative activity and receptor affinity. J Med Chem 50:2682–2692
45. **Overk CR, Peng KW, Asghodom RT, Kastrati I, Lantvit DD, Qin Z, Frasor J, Bolton JL, Thatcher GRJ** 2007 Structure-activity relationships for a family of benzothiophene selective estrogen receptor modulators including raloxifene and arzoxifene. ChemMedChem 2:1520–1526
46. **Taguchi Y, Koslowski M, Bodenner D** 2004 Binding of estrogen receptor with estrogen conjugated to bovine serum albumin (BSA). Nucl Rec 2:5
47. **Stevis PE, Deecher DC, Suhadolnik L, Mallis LM, Frail DE** 1999 Differential effects of estradiol and estradiol-BSA conjugates. Endocrinology 140:5455–5458
48. **Qiu J, Bosch MA, Tobias SC, Grandy DK, Scanlan TS, Ronnekleiv OK, Kelly MJ** 2003 Rapid signaling of estrogen in hypothalamic neurons involves a novel G-protein-coupled estrogen receptor that activates protein kinase C. J Neurosci 23:9529–9540
49. **Marino M, Galluzzo P, Leone S, Acconcia F, Ascenzi P** 2006 Nitric oxide impairs the 17β-estradiol-induced apoptosis in human colon adenocarcinoma cells. Endocr Relat Cancer 13:559–569
50. **Ciani E, Virgili M, Contestabile A** 2002 Akt pathway mediates a cGMP-dependent survival role of nitric oxide in cerebellar granule neurones. J Neurochem 81:218–228
51. **Fraser M, Chan SL, Chan SS, Fiscus RR, Tsang BK** 2006 Regulation of p53 and suppression of apoptosis by the soluble guanylyl cyclase/cGMP pathway in human ovarian cancer cells. Oncogene 25:2203–2212
52. **Chan SL, Fiscus RR** 2003 Guanylyl cyclase inhibitors NS2028 and ODQ and protein kinase G (PKG) inhibitor KT5823 trigger apoptotic DNA fragmentation in immortalized uterine epithelial cells: anti-apoptotic effects of basal cGMP/PKG. Mol Hum Reprod 9:775–783
53. **Kim YM, Chung HT, Kim SS, Han JA, Yoo YM, Kim KM, Lee GH, Yun HY, Green A, Li J, Simmons RL, Billiar TR** 1999 Nitric oxide protects PC12 cells from serum deprivation-induced apoptosis by cGMP-dependent inhibition of caspase signaling. J Neurosci 19:6740–6747
54. **Gross A, McDonnell JM, Korsmeyer SJ** 1999 BCL-2 family members and the mitochondria in apoptosis. Genes Dev 13:1899–1911
55. **Fiskum G** 2000 Mitochondrial participation in ischemic and traumatic neural cell death. J Neurotrauma 17:843–855
56. **Kim JS, Ohshima S, Pediaditakis P, Lemasters JJ** 2004 Nitric oxide: a signaling molecule against mitochondrial permeability transition- and pH-dependent cell death after reperfusion. Free Radic Biol Med 37:1943–1950
57. **Chen F, Arseven OK, Cryns VL** 2004 Proteolysis of the mismatch repair protein MLH1 by caspase-3 promotes DNA damage-induced apoptosis. J Biol Chem 279:27542–27548
58. **Soule HD, Maloney TM, Wolman SR, Peterson Jr WD, Brenz R, McGrath CM, Russo J, Pauley RJ, Jones RF, Brooks SC** 1990 Isolation and characterization of a spontaneously immortalized human breast epithelial cell line, MCF-10. Cancer Res 50:6075–6086
59. **Spink DC, Spink BC, Cao JQ, DePasquale JA, Pentecost BT, Fasco MJ, Li Y, Sutter TR** 1998 Differential expression of CYP1A1 and CYP1B1 in human breast epithelial cells and breast tumor cells. Carcinogenesis 19:291–298
60. **Allred DC, Brown P, Medina D** 2004 The origins of estrogen receptor α-positive and estrogen receptor α-negative human breast cancer. Breast Cancer Res 6:240–245
61. **Chen JQ, Russo J** 2009 ERα-negative and triple negative breast cancer: molecular features and potential therapeutic approaches. Biochim Biophys Acta 1796:162–175
62. **Adam L, Crépin M, Isräel L** 1997 Tumor growth inhibition, apoptosis, and Bcl-2 down-regulation of MCF-7ras tumors by sodium phenylacetate and tamoxifen combination. Cancer Res 57:1023–1029
63. **Somaï S, Chaouat M, Jacob D, Perrot JY, Rostène W, Forgez P, Gompel A** 2003 Antiestrogens are pro-apoptotic in normal human breast epithelial cells. Int J Cancer 105:607–612
64. **Lewis-Wambi JS, Jordan VC** 2009 Estrogen regulation of apoptosis: how can one hormone stimulate and inhibit? Breast Cancer Res 11:206
65. **Boyan BD, Sylvia VL, Frambach T, Lohmann CH, Dietl J, Dean DD, Schwartz Z** 2003 Estrogen-dependent rapid activation of protein kinase C in estrogen receptor-positive MCF-7 breast cancer cells and estrogen receptor-negative HCC38 cells is membrane-mediated and inhibited by tamoxifen. Endocrinology 144:1812–1824
66. **Helguero LA, Faulds MH, Gustafsson JA, Haldosén LA** 2005 Estrogen receptors α (ERα) and β (ERβ) differentially regulate proliferation and apoptosis of the normal murine mammary epithelial cell line HC11. Oncogene 24:6605–6616
67. **Tsai EM, Wang SC, Lee JN, Hung MC** 2001 Akt activation by estrogen in estrogen receptor-negative breast cancer cells. Cancer Res 61:8390–8392
68. **Levin ER** 2001 Cell localization, physiology, and nongenomic actions of estrogen receptors. J Appl Physiol 91:1860–1867
69. **Prossnitz ER, Maggiolini M** 2009 Mechanisms of estrogen signaling and gene expression via GPR30. Mol Cell Endocrinol 308:32–38
70. **Rubanyi GM, Freay AD, Kauser K, Sukovich D, Burton G, Lubahn DB, Couse JF, Curtis SW, Korach KS** 1997 Vascular estrogen receptors and endothelium-derived nitric oxide production in the

mouse aorta. Gender difference and effect of estrogen receptor gene disruption. J Clin Invest 99:2429–2437

71. **Weiner CP, Lizasoain I, Baylis SA, Knowles RG, Charles IG, Moncada S** 1994 Induction of calcium-dependent nitric oxide synthases by sex hormones. Proc Natl Acad Sci USA 91:5212–5216
72. **García-Durán M, de Frutos T, Díaz-Recasens J, García-Gálvez G, Jiménez A, Montón M, Farré J, Sánchez de Miguel L, González-Fernández F, Arriero MD, Rico L, García R, Casado S, López-Farré A** 1999 Estrogen stimulates neuronal nitric oxide synthase protein expression in human neutrophils. Circ Res 85:1020–1026
73. **Simoncini T, Hafezi-Moghadam A, Brazil DP, Ley K, Chin WW, Liao JK** 2000 Interaction of oestrogen receptor with the regulatory subunit of phosphatidylinositol-3-OH kinase. Nature 407:538–541
74. **Lu Q, Pallas DC, Surks HK, Baur WE, Mendelsohn ME, Karas RH** 2004 Striatin assembles a membrane signaling complex necessary for rapid, nongenomic activation of endothelial NO synthase by estrogen receptor α. Proc Natl Acad Sci USA 101:17126–17131
75. **Acconcia F, Totta P, Ogawa S, Cardillo I, Inoue S, Leone S, Trentalance A, Muramatsu M, Marino M** 2005 Survival versus apoptotic 17β-estradiol effect: role of ERα and ERβ activated non-genomic signaling. J Cell Physiol 203:193–201
76. **Prueitt RL, Boersma BJ, Howe TM, Goodman JE, Thomas DD, Ying L, Pfiester CM, Yfantis HG, Cottrell JR, Lee DH, Remaley AT, Hofseth LJ, Wink DA, Ambs S** 2007 Inflammation and IGF-I activate the Akt pathway in breast cancer. Int J Cancer 120:796–805
77. **Kim PK, Zamora R, Petrosko P, Billiar TR** 2001 The regulatory role of nitric oxide in apoptosis. Int Immunopharmacol 1:1421–1441
78. **Li J, Billiar TR, Talanian RV, Kim YM** 1997 Nitric oxide reversibly inhibits seven members of the caspase family via S-nitrosylation. Biochem Biophys Res Commun 240:419–424
79. **Li J, Yang S, Billiar TR** 2000 Cyclic nucleotides suppress tumor necrosis factor α-mediated apoptosis by inhibiting caspase activation and cytochrome c release in primary hepatocytes via a mechanism independent of Akt activation. J Biol Chem 275:13026–13034
80. **Kim YM, Talanian RV, Billiar TR** 1997 Nitric oxide inhibits apoptosis by preventing increases in caspase-3-like activity via two distinct mechanisms. J Biol Chem 272:31138–31148
81. **Estévez AG, Spear N, Thompson JA, Cornwell TL, Radi R, Barbeito L, Beckman JS** 1998 Nitric oxide-dependent production of cGMP supports the survival of rat embryonic motor neurons cultured with brain-derived neurotrophic factor. J Neurosci 18:3708–3714
82. **Frame S, Cohen P** 2001 GSK3 takes centre stage more than 20 years after its discovery. Biochem J 359:1–16
83. **Kim JS, Ohshima S, Pediaditakis P, Lemasters JJ** 2004 Nitric oxide protects rat hepatocytes against reperfusion injury mediated by the mitochondrial permeability transition. Hepatology 39:1533–1543
84. **Nisoli E, Clementi E, Paolucci C, Cozzi V, Tonello C, Sciorati C, Bracale R, Valerio A, Francolini M, Moncada S, Carruba MO** 2003 Mitochondrial biogenesis in mammals: the role of endogenous nitric oxide. Science 299:896–899
85. **Kim JS, Nitta T, Mohuczy D, O'Malley KA, Moldawer LL, Dunn Jr WA, Behrns KE** 2008 Impaired autophagy: a mechanism of mitochondrial dysfunction in anoxic rat hepatocytes. Hepatology 47:1725–1736
86. **Green DR** 2005 Apoptotic pathways: ten minutes to dead. Cell 121:671–674
87. **Chen JQ, Brown TR, Yager JD** 2008 Mechanisms of hormone carcinogenesis: evolution of views, role of mitochondria. Adv Exp Med Biol 630:1–18
88. **Martin JH, Alalami O, van den Berg HW** 1999 Reduced expression of endothelial and inducible nitric oxide synthase in a human breast cancer cell line which has acquired estrogen independence. Cancer Lett 144:65–74